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THE CRYSTALLIZATION OF ACETYLCHOLINESTERASE (AChE)
FROM TORPEDO ELECTRIC ORGAN

ANNUAL REPORT

I. SILMAN
J.L. SUSSMAN
June 25, 1988

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Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012
Contract No. DAMD17-87-C-7003

Depts. of Neurobiology and Structural Chemistry
Weizmann Institute of Science
Rehovot 76100, Israel

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<p>The objective of this project is the crystallization of the enzyme acetylcholinesterase (AChE), with the long-term objective of determining its three-dimensional structure and, thereby, the detailed topography of its active site.</p> <p><u>Torpedo</u> electric organ was selected since it is a rich source of AChE and possesses an amino acid sequence very similar to that of mammalian AChE. A dimeric form of this enzyme was purified by a procedure which involved selective solubilization with a phosphatidyl-inositol-specific phospholipase C of bacterial origin, followed by affinity chromatography employing a Sepharose conjugate of a suitable quaternary affinity ligand. A highly purified AChE preparation was obtained in amounts which permitted a systematic attempt to crystallize the enzyme.</p> <p>In order to obtain a crystal form of the AChE preparation suitable for high-resolution X-ray studies, we examined hundreds of different crystallization conditions. As a result we ...</p>				
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19. ABSTRACT (continued)

were able to obtain two different crystal forms which diffract to better than 3 Å resolution. One of these forms, which was obtained from ammonium sulfate-phosphate buffer, is capable of being "shock-cooled" to liquid nitrogen temperatures, which permitted X-ray data to be collected at this temperature. This "shock-cooling" procedure prolongs the lifetime of the crystal in the X-ray beam almost indefinitely and has already permitted us to collect a preliminary set of three-dimensional X-ray data. We are thus in a position to proceed with the determination of the three-dimensional structure of AChE.

SUMMARY

The objective of this proposal is the crystallization of acetylcholinesterase (AChE) from *Torpedo* electric organ, with the long-term goal of determining the three-dimensional structure of the enzyme and, thereby, the detailed topography of its catalytic site.

Torpedo electric organ was selected since it is a rich source of AChE and possesses an amino acid sequence very similar to that of mammalian AChE. A dimeric form of this enzyme was purified by a procedure which involved selective solubilization with a phosphatidylinositol-specific phospholipase C (PIPLC) of bacterial origin, followed by affinity chromatography employing a Sepharose conjugate of a suitable quaternary affinity ligand. A highly purified AChE preparation was obtained in amounts which permitted a systematic attempt to crystallize the enzyme.

In order to obtain a crystal form of the AChE preparation suitable for high-resolution X-ray studies, we examined hundreds of different crystallization conditions. As a result we were able to obtain two different crystal forms which diffract to better than 3 Å resolution. One of these forms, which was obtained from ammonium sulfate in phosphate buffer, is capable of being "shock-cooled" to liquid nitrogen temperatures and permits X-ray data to be collected at this temperature. This "shock-cooling" procedure prolongs the lifetime of the crystal in the X-ray beam almost indefinitely and has already permitted us to collect a preliminary set of X-ray data up to 5 Å resolution. We are thus in a position to proceed with the determination of the three-dimensional structure of AChE.

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OBJECTIVES

The objective of this proposal was the crystallization of acetylcholinesterase (AChE) from *Torpedo* electric organ, with the long-term intention of determining the three-dimensional structure of the enzyme and, thereby, the detailed topography of its catalytic site.

BACKGROUND

In our original proposal we developed a rationale for the selection of an AChE preparation suitable for attempts at growing crystals for use in the determination of the three-dimensional structure of this enzyme by X-ray crystallography. We chose *Torpedo* electric organ as the source of the enzyme for three principal reasons:

1. Electric organ tissue of *Torpedo californica* contains high concentrations of AChE and the tissue is readily available from commercial sources in kilogram quantities. Thus we considered it to be an excellent source for the amounts of highly purified AChE required for a project aimed at crystallization and eventual determination of the three-dimensional structure of AChE.
2. The amino acid sequence of *Torpedo* AChE was determined both by DNA cloning (1) and by direct sequencing (2) and the arrangement of its intrasubunit disulfide bonds was also established (3). It appears to possess a high degree of homology, with respect to both sequence and disulfide bond arrangement, with both fetal calf serum AChE (B.P. Doctor, personal communication) and human serum cholinesterase (4). Thus, information concerning its three-dimensional structure in general, and its active site in particular, should be highly pertinent to understanding the mode of action of the human enzyme and in devising therapeutic and prophylactic approaches to poisoning by organophosphorus and carbamate nerve agents.
3. We have characterized a dimeric form of AChE from *Torpedo* electric organ which is bound to the plasma membrane via the diglyceride moiety of covalently attached phosphatidylinositol (PI) (Fig. 1) (5, 6). In this dimer the PI is attached to the COOH-terminus of each catalytic subunit through an intervening oligosaccharide sequence, which is apparently added post-translationally, and the diglyceride moiety of the PI serves as the hydrophobic anchor (7). The AChE dimer thus belongs to a recently described class of membrane proteins which are so attached to the plasma membrane (8). Such PI-anchored proteins can often be solubilized selectively by a PI-specific phospholipase C of bacterial origin (8), and this is also the case for the *Torpedo* AChE dimer (9), thus allowing subsequent purification of the enzyme with minimum "lytic" damage or denaturation. For crystallographic studies, this preparation should be preferable to preparations obtained by proteolytic digestion of the asymmetric forms of AChE from either *Torpedo* or *Electrophorus*, which yield an asymmetric tetramer which has undergone considerable proteolytic "nicking" (10). This is because "nicking" gives rise to a heterogeneity which may prove detrimental to crystal growth and may also hinder interpretation of the X-ray data if nicked and non-nicked forms coexist in the same crystal.

As described below, we have been able, during the period covered by this Annual Report, to develop a procedure for reproducible and routine production of amounts of *Torpedo* electric organ AChE suitable for scanning a variety of crystallization conditions. We have, as a result, been able to obtain two crystal forms of the enzyme which diffract to a high resolution and have permitted us to collect preliminary crystallographic data. We are now in a position to proceed directly to studies leading to the solution of the three-dimensional structure of this form of AChE.

RESULTS AND DISCUSSION

I. Purification of AChE

Purification of AChE was carried out by an affinity chromatography procedure, subsequent to its solubilization by PIPLC from *Staphylococcus aureus*. The PIPLC, which is purified to homogeneity from the culture medium of the bacterium (11), was supplied by Dr. M.G. Low (Dept. of Physiology and Cellular Biophysics, College of Physicians and Surgeons of Columbia University).

The particulate fraction from a homogenate of frozen electric organ tissue from *Torpedo californica* (obtained from Pacific Biomarine, Venice, CA) suspended in 0.1 M NaCl 0.01 M Tris, pH 8.0, was exposed to PIPLC (1 µg/ml) for 18 hr at room temperature. As reported previously (9), almost quantitative solubilization of the G₂ AChE dimer was thus obtained. After high speed centrifugation at 78,000 g for 1 hr, at 4°C, the supernatant was passed over an affinity chromatography column (bed volume ca. 7 ml), consisting of the affinity ligand (m-aminophenyl)trimethylammonium, coupled to Sepharose 2B via a dicaproyl spacer (5, 12). After extensive washing, the bound AChE was eluted from the column with 2 mM decamethonium bromide in the application buffer. The purified enzyme was then dialyzed exhaustively against 0.1 M NaCl 0.01% sodium azide 1 mM 2[N-morpholino]ethanesulfonic acid (MES), pH 6.5 and concentrated up to 10-15 mg/ml in a Centricon 30 microconcentrator (Amicon Co., Lexington, MA). The purified AChE displayed one major polypeptide band, of apparent molecular weight 65,000, on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of reducing agent. In the absence of reducing agent it migrated as a species of apparent molecular weight 130,000, corresponding to the disulfide-linked subunit dimer (Fig. 2). It displayed a catalytic activity of >2800 units per mg protein, using 3 mM [³H]acetylcholine as substrate at pH 7.4 at 25°C. About 7 mg of purified AChE could be obtained routinely from ca. 450 g of electric organ tissue.

We have recently subjected this affinity-purified AChE preparation to an additional purification step by high performance liquid chromatography (HPLC); this involves absorption on a Mono-Q anion exchange column (Pharmacia, Uppsala, Sweden), followed by elution with a linear NaCl gradient. The enzyme is loaded on the HPLC column at low ionic strength and eluted with a 0-0.8 M NaCl gradient. The bulk of the enzyme is eluted as a single major peak at ca. 0.72 M NaCl (Fig. 3). It is, however, always preceded by a minor peak, eluting at ca. 0.72 M salt, accounting for ca. 5% of both the protein and enzymic activity. This minor peak appears indistinguishable from the principal peak on SDS-PAGE. A plausible explanation for its significance is that it corresponds to an immature form of the enzyme, lacking a negatively charged sugar epitope, the situation being similar to that recently proposed for the G₂ dimer in electric organ tissue of *Torpedo marmorata* by Bon et al. (13). We are currently investigating whether removal of this minor peak by the HPLC procedure improves the reproducibility of crystal production and/or the quality of the crystals obtained.

II. Crystallization

Attempts to crystallize *Torpedo californica* AChE were performed using the hanging drop method (14-16). This crystallization method utilizes the slow equilibration of concentration between a small drop containing the protein in dilute solution of the precipitating agent and a large volume of reservoir containing the same precipitating agent at a higher concentration. Sealing of the protein drop and the reservoir from the outside atmosphere permits fine controlling of precipitation conditions. Screening of a wide variety of precipitation conditions, while using small quantities of protein, is made possible since the volume of each drop is 5-10 µl and thus contains, at a final AChE concentration of ca. 5 mg/ml, a total of 25-50 µg protein.

The screening of AChE crystallization conditions involved several stages. The first stage consisted of testing the most commonly used protein-precipitating agents. These include inorganic salts and various alcohols. The next stage involved the combination of various precipitating agents such as mixtures of different salts and of salts with various alcohols. Microcrystals of AChE were obtained under various crystallization conditions, but our first success in growing large single crystals of AChE of dimensions suitable for X-ray data collection, was achieved when using low molecular weight polyethylene glycol (PEG) as the precipitant. The crystals grew to 0.2-0.3 mm in size, which is somewhat small unless the crystal diffraction is especially strong or the data are collected using a particularly strong X-ray source.

Since the AChE crystals diffracted weakly, efforts were directed at obtaining larger ones. The technique used for increasing crystal size was that of seeding. In this technique a small or medium sized crystal, which has stopped growing spontaneously due to accumulation of imperfections or impurities on its surface, is transferred to a solution containing a slightly less concentrated precipitating agent. This solution is swirled gently around the crystal causing its outer surface to dissolve and thus exposing an uncontaminated fresh crystal surface. The seed crystal is then transferred to a fresh protein hanging drop where its clean faces can form an ideal nucleating environment for further crystal growth.

This technique of seeding was used on AChE crystals growing in hanging drops containing 12 mg/ml protein, 30% PEG 200 and 50 mM MES buffer, pH 6.0. Crystals 0.4 mm in size grew within 3 weeks from seed crystals which were 0.2 mm in size. The crystal cell parameters of this form were determined via X-ray studies using a Rigaku AFC5-R rotating anode diffractometer operated at 10 kW. The crystals were found to be orthorhombic, space group $P222_1$, with $a = 163.4(\pm 0.2)$ Å, $b = 112.1(\pm 0.2)$ Å and $c = 81.3(\pm 0.1)$ Å (Fig. 4). Assuming the unit cell to contain 4 AChE dimers, we calculated the ratio of the volume of the asymmetric unit to the molecular weight of the dimer (130,000) to be $V_m = 2.95 \text{ \AA}^3/\text{dalton}$, well within the range outlined by Matthews (17). The crystals diffract to 2.0 Å resolution and lose ca. 25% of their diffracting power within 24 hr in the X-ray beam at room temperature. An attempt to prevent the decay in crystalline order (which is caused by X-ray irradiation), by cooling the crystals to -150°C, resulted in total loss of diffracting power. The procedure for purification and crystallization as well as the preliminary X-ray results have just been published (18).

Having found conditions for growing single crystals of AChE which diffract to high resolution we embarked on exploring conditions to prolong the lifetime of the irradiated crystals in the X-ray beam. We sought, therefore, to grow a different crystal form which would be either less sensitive to X-ray irradiation or could survive the shock-cooling technique.

A method which has proved very successful for preserving the lifetime of a protein crystal under X-ray irradiation involves the shock-cooling of the crystal down to the temperature of boiling N₂ (ca. -150°C), followed by collection of diffraction data at this temperature. This method, which was developed for small molecule crystallography (19) and was extended in our laboratory to work with biological macromolecules (20), entails coating the crystal with a film of nonaqueous, viscous material such as oil, picking it up on a glass fiber by adhesion and transferring the fiber to the goniometer head in a position where a constant stream of boiling N₂ gas is flowing. The advantages of this method are:

- 1) There is virtually no decay in the diffraction pattern after 2 weeks or more of X-ray irradiation.
- 2) The frozen crystal does not slip during data collection.
- 3) Corrections to diffraction intensities due to absorption of X-rays by the glass capillary and the mother liquor, in which crystals are mounted for room temperature data collection, are minimized to

only those corrections due to the crystal shape.

4) The thermal motion of atoms in the crystal is reduced, thus producing better data and easier interpretation of the electron density map.

Use of the shock-cooling technique permits collection of an entire data set from just one crystal, thus avoiding errors arising out of the necessity of having to scale data sets from several crystals. No every crystal can withstand shock-cooling without being shattered and the experience accumulated in our lab shows that about 70% of the biological macromolecules survive it. Usually crystals grown from inorganic salt solutions survive it better than those grown from organic precipitants. As a consequence, we embarked on trying to grow AChE crystals from a wide combination of inorganic salts. Table I shows a list of crystallization conditions tested.

After numerous attempts we were finally successful in crystallizing the AChE preparation from $(\text{NH}_4)_2\text{SO}_4$ in the presence of either 0.2 or 0.36 M phosphate buffer, pH 7.0. Crystals grew within 2 months to a size of 0.6 mm and diffracted to 2.5 Å resolution (Fig. 5). The crystal cell parameters of this form were determined by X-ray diffraction at -150°C using a Rigaku AFC5-R rotating anode diffractometer operated at 10 kW. The crystals were found to be hexagonal, space group P6₂22 or P6₄22, with $a = b = 110.40(\pm 0.1)$ Å, $c = 136.95(\pm 0.06)$ Å. Assuming the unit cell to contain 6 AChE dimers we calculate the ratio of the volume of the asymmetric unit to the molecular weight of the monomer (65,000) to be $V_m = 1.85 \text{ \AA}^3/\text{dalton}$.

Using the shock-cooling technique on the crystals grown from ammonium sulfate, preliminary diffraction data set of 2339 reflections was collected out to 5 Å resolution at -150°C. The two monitor reflections showed no decay in diffracting power over 21 hr of open shutter time (ca. 36 hr from the start of irradiation). Data were collected at a scanning motor rate of 4°/min so as to ensure good statistics for the weak reflections.

The atomic structure of a molecule can be traced from an electron density map based on single crystal X-ray diffraction data. In order to calculate such a map, one needs to sum the structure factors (F) of all the reflections at any point in the map. These F s are complex numbers whose magnitude is related to the intensity of the reflection but their phases are unknown. In solving the atomic structure of small molecules one can use statistical relationships between intensities (i.e., direct methods). However, a similar direct computation approach for a protein molecule is not possible. The approach for finding the phases of the F s in crystals of biological macromolecules involves the use of isomorphous heavy atom derivatives. A good heavy atom derivative is formed when a heavy atom is soaked into a native crystal and attaches in such a way as to create observable changes in the diffraction pattern without perturbing the structure of the native molecule. From the differences between the F s of the native and the derivative one can solve for the position of the heavy atom and hence calculate its phase contribution to the F s of the heavy atom derivative. From data collected from two or more derivatives, the phase of the native molecule can be derived directly (with estimates of the errors for each phase determined) thus permitting the calculation of an electron density map. We have now initiated a search for isomorphous heavy atom derivatives which will be used to solve the phase problem in order to determine the 3-dimensional structure of the AChE molecule.

CONCLUSIONS

The work carried out during the first year of this project has justified the rationale which served as its basis. Thus the choice of the enzyme preparation, namely the dimeric form of AChE solubilized from electric organ tissue of *Torpedo californica* by PI-specific phospholipase C, has permitted us to conveniently obtain large amounts of intact, highly purified AChE. This, in turn, has led to successful crystallization experiments which have produced two crystal forms of the enzyme which diffract to high resolution. One crystal form, grown from ammonium sulfate-phosphate solutions, withstands shock-cooling to liquid nitrogen temperatures, and diffracts without irradiation decay at a temperature of -150°C. The way is thus open to the solution of the three-dimensional structure of *Torpedo* AChE.

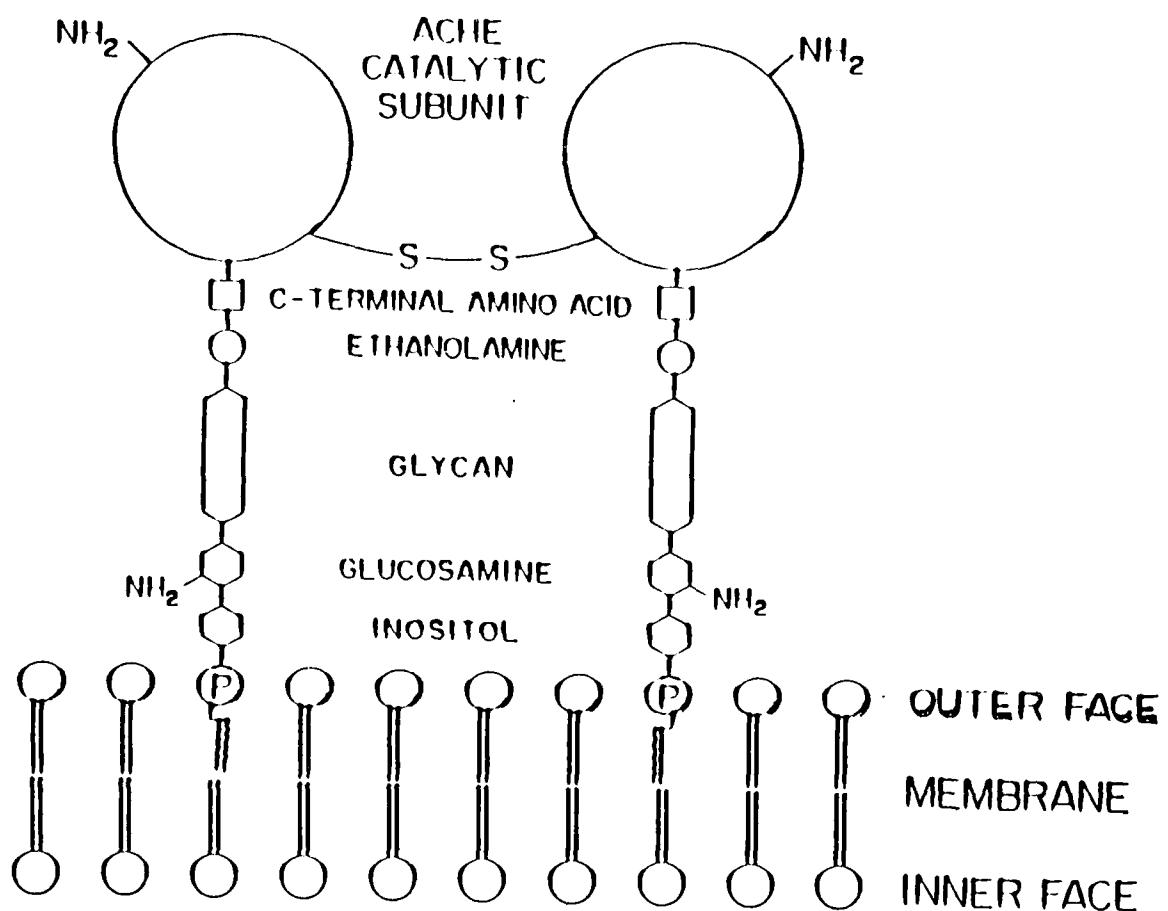


Fig. 1: Schematic representation of the phosphatidylinositol-anchored dimer of AChE from *Torpedo californica*.

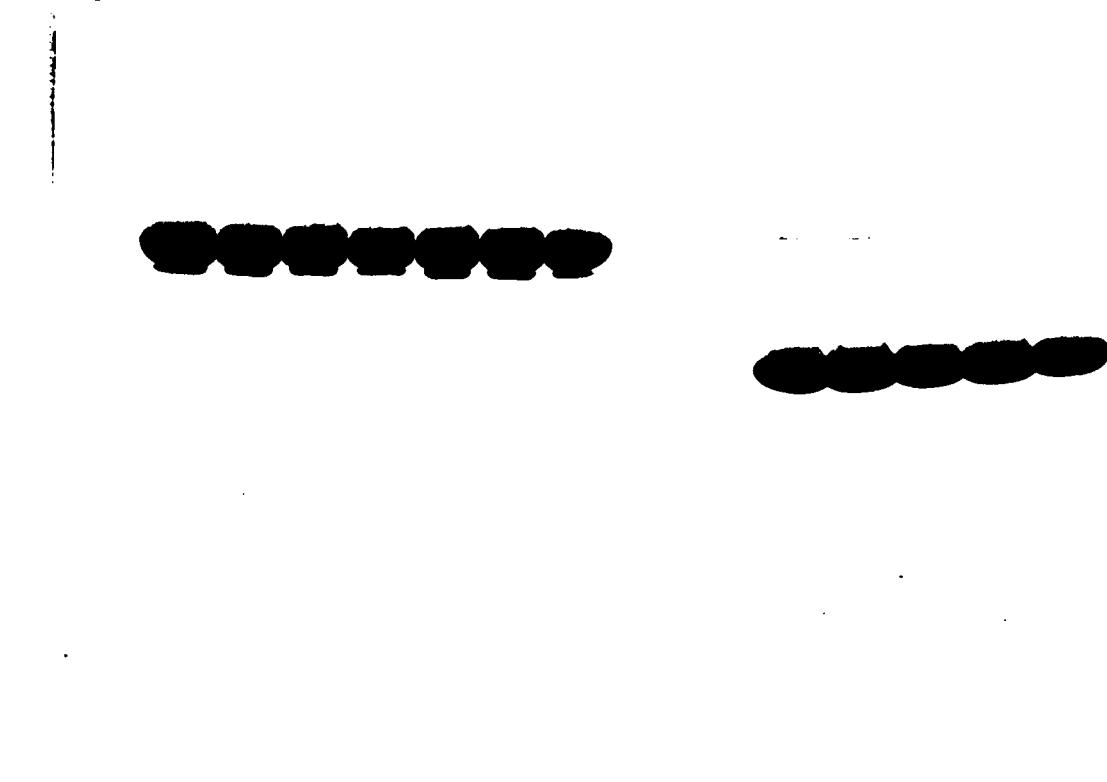


Fig. 2: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of AChE from *Torpedo* electric organ purified by affinity chromatography subsequent to solubilization with PIPLC.

Staining was with Coomassie Brilliant Blue. The consecutive lanes correspond to consecutive fractions obtained from the affinity column. The series on the left were electrophoresed in the absence of reducing agent and show the intact, disulfide-linked dimer. The series on the right show the catalytic subunit monomer obtained in the presence of reducing agent. The constancy of the electrophoresis patterns across the elution profile suggests that the minor components observed, which are only seen on heavily overloaded gels, result from the presence of small amounts of "nicked" catalytic subunit polypeptides.

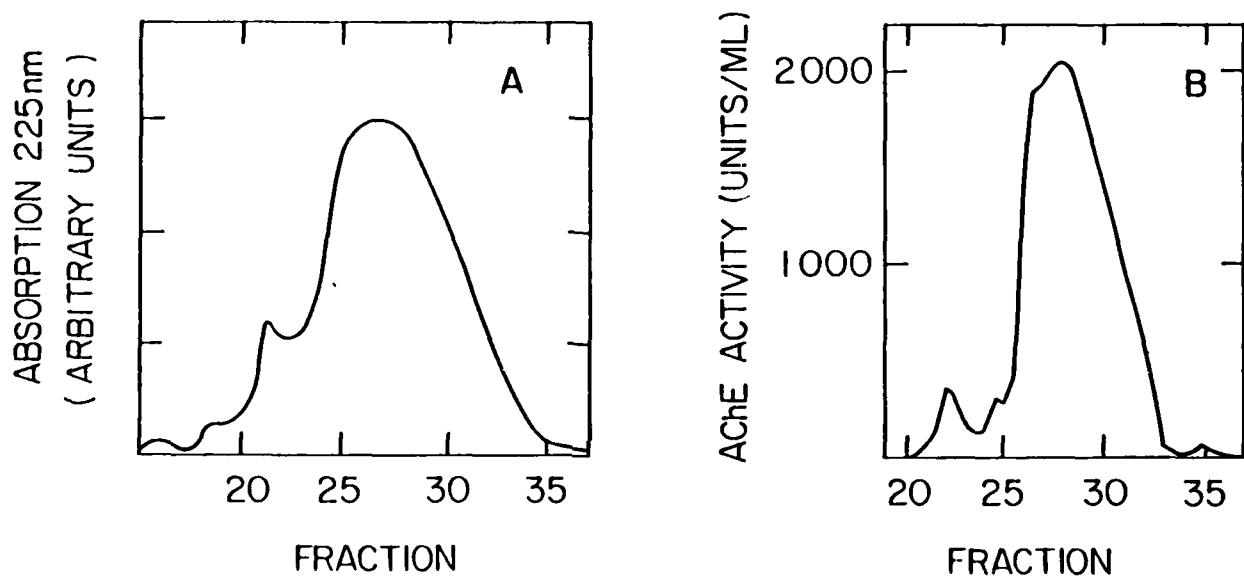


Fig. 3: Fractionation by high performance liquid chromatography (HPLC) of the *Torpedo* AChE dimer subsequent to purification by affinity chromatography.

HPLC was performed on a Mono-Q anion exchange column (Pharmacia). The AChE was applied in 20 mM Tris chloride, pH 8.0 (ca. 10 mg of purified enzyme in 500 microliters of buffer); it was eluted with a linear gradient of 0-0.8 M NaCl in the same buffer. Fractions of 0.5 ml were collected. The main peak of AChE was eluted at ca. 0.72 M NaCl and the small shoulder which preceded it was eluted at ca. 0.56 M NaCl. A) Elution profile obtained by continuous flow monitoring of protein absorption at 225 nm. B) Elution profile obtained by monitoring AChE activity of individual fractions by the Ellmann procedure.

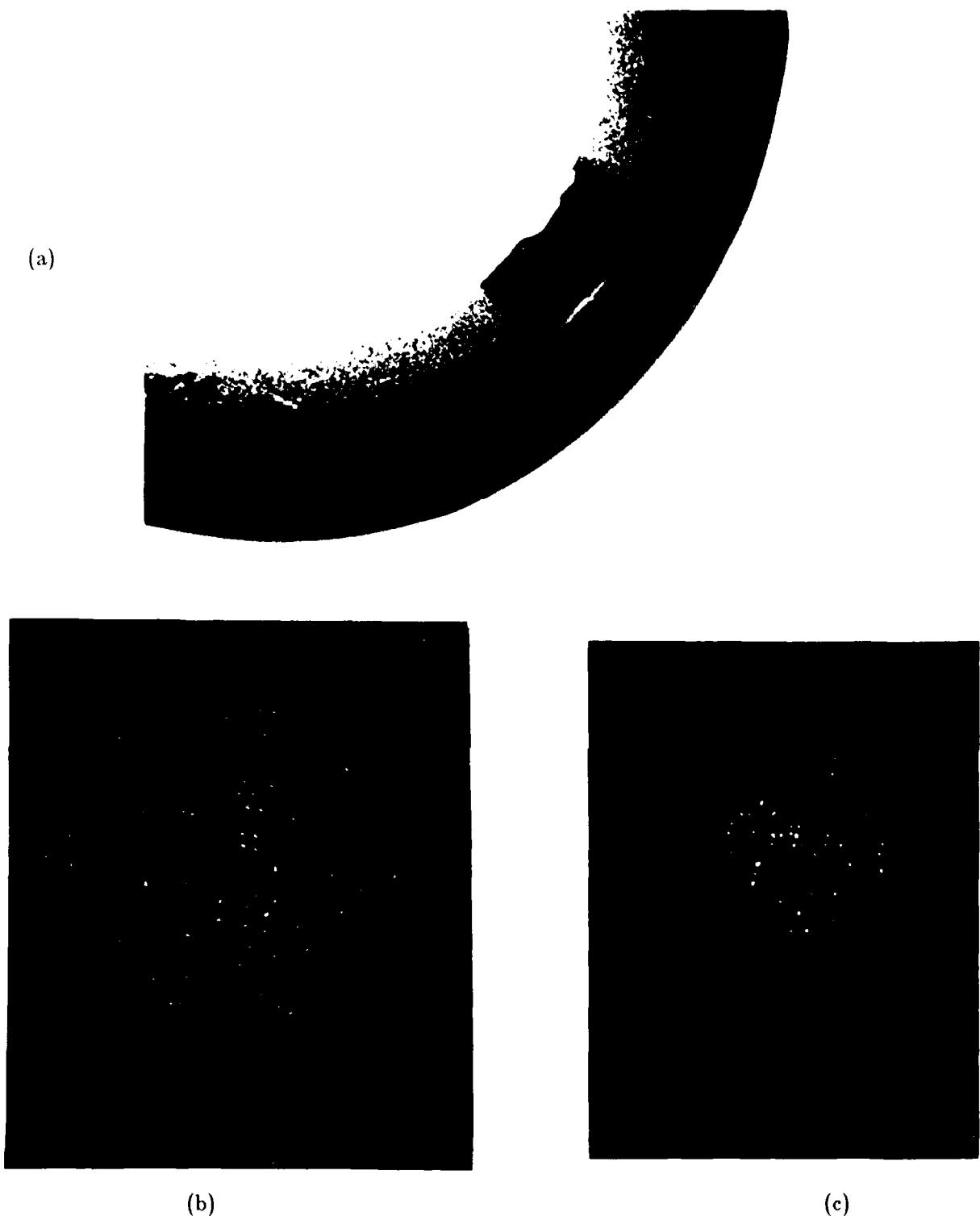
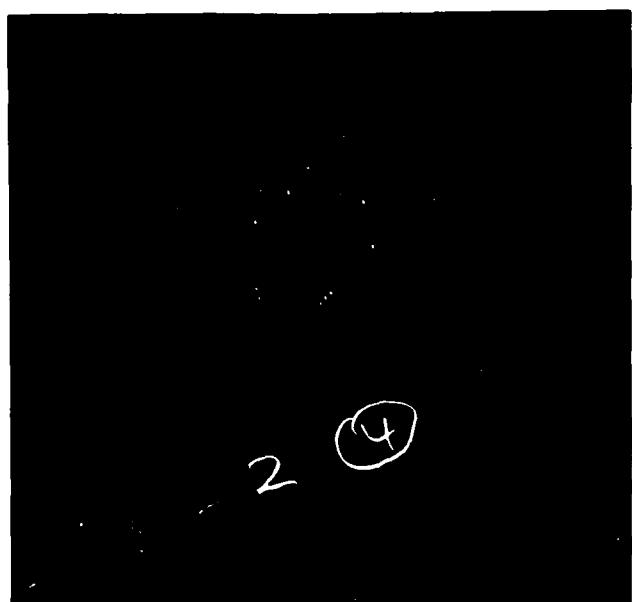
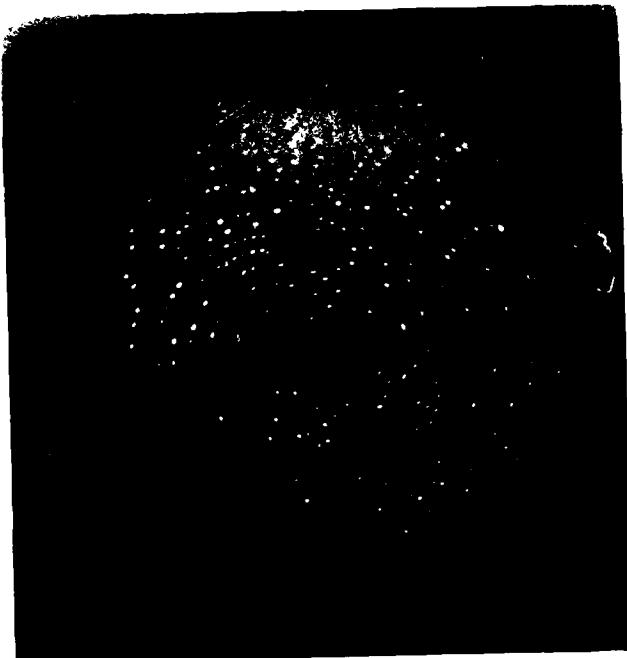
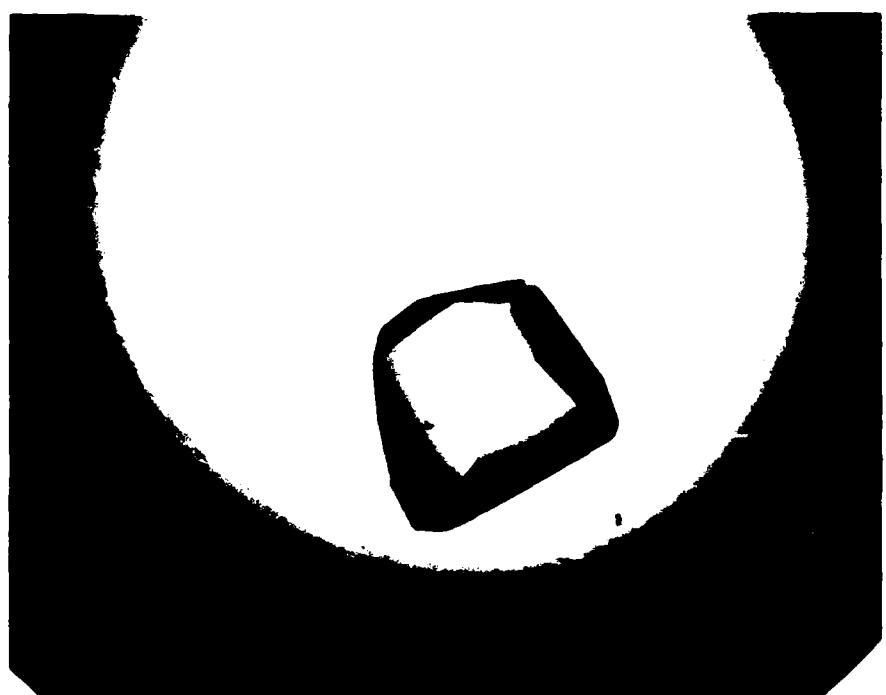


Fig. 4: AChE crystals and X-ray diffraction from PEG 200.

- (a) AChE crystals grown from PEG 200 (magnification X50).
- (b) Still X-ray diffraction from crystal grown from PEG 200.
- (c) Precession X-ray diffraction from the same crystal.



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2
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Table 1: AChE Crystallization Conditions

no.	drop	reservoir	pH	results
1	35% AS	45% AS	7.0	-
2	40% AS	50% AS	7.0	-
3	45% AS	55% AS	7.0	-
4	30% AS	40% AS	6.6	-
5	25% AS	35% AS	6.6	-
6	20% AS	30% AS	6.6	-
7	25% AS	35% AS	6.9	-
8	50% AS	60% AS	6.6	-
9	55% AS	65% AS	6.6	-
10	45% AS	55% AS	5.4	-
11	45% AS	55% AS	6.0	-
12	45% AS	55% AS	6.9	-
13	40% AS, 0.086 M phosphate	50% AS	7.4	-
14	40% AS, 0.086 M phosphate	50% AS	7.0	-
15	40% AS, 0.086 M phosphate	50% AS	6.6	-
16	45% AS, 0.079 M phosphate	55% AS	6.6	-
17	45% AS, 0.079 M phosphate	55% AS	7.0	-
18	27% AS, 0.36 M phosphate	50% AS	7.0	-
19	27% AS, 0.36 M phosphate	60% AS	7.0	large trigonal crystal
20	40% AS, 0.33 M phosphate	60% AS	7.0	large trigonal crystal
21	40% AS, 0.1 M phosphate	60% AS	7.0	large trigonal crystal
22	27% AS, 0.36 M phosphate	60% AS	7.0	0.7 mm crystal, P6 ₂ 22, LT
23	24% MPD, 100 mM NaCl	34% MPD	6.6	-
24	21% MPD, 100 mM NaCl	31% MPD	6.6	-
25	16% MPD, 100 mM NaCl	26% MPD	6.6	-
26	13% MPD, 100 mM NaCl	23% MPD	6.6	-
27	10% MPD, 100 mM NaCl	20% MPD	6.6	-
28	7% MPD, 100 mM NaCl	17% MPD	6.6	-
29	25% PEG 200	50% PEG	6.6	small crystal
30	25% PEG 200	50% PEG	5.6	small crystal
31	25% PEG 200	40% PEG	6.0	small crystal
32	15% PEG 200	30% PEG	6.0	.38 mm crystal, P222 ₁ , RT
33	20% PEG 400	40% PEG	6.6	small plates
34	14% PEG 400, 0.014M spermine	40% PEG	5.6	small hexagons
35	7% PEG 600	15% PEG	6.6	-
36	7% PEG 600	15% PEG	5.6	-
37	4% PEG 1000	8% PEG	6.6	-
38	4% PEG 1000	8% PEG	5.6	-
39	2% PEG 1500	4% PEG	6.6	-
40	10% PEG 1500	20% PEG	6.6	rods
41	4% PEG 3350	6% PEG	6.6	-
42	2.86% PEG 3350	5% PEG	6.6	-
43	1.6% PEG 3350	3% PEG	6.6	-
44	5% PEG 3350, 1 M NaCl	10% PEG	6.6	-
45	2% PEG 3350, 1.5 M NaCl	5% PEG	5.4	-
46	2% PEG 3350, 1.5 M NaCl	5% PEG	6.0	-

Table 1 (cont.)

no.	drop	reservoir	pH	results
47	1.5% PEG 6000	3% PEG	6.6	-
48	1% PEG 6000	2% PEG	6.6	-
49	0.75% PEG 6000	2% PEG	6.6	-
50	5% PEG 6000, 1 M NaCl	10% PEG	6.6	-
51	5% PEG 6000, 1.5 M NaCl	10% PEG	6.6	-
52	5% PEG 6000, 1.5 M NaCl	10% PEG	6.0	-
53	5% PEG 6000, 1.5 M NaCl	10% PEG	5.4	-
54	2% PEG 6000, 1.5 M NaCl	5% PEG	6.6	-
55	2% PEG 6000, 1.5 M NaCl	5% PEG	6.0	-
56	2% PEG 6000, 1.5 M NaCl	5% PEG	5.4	-
57	5% PEG 6000, 0.5 M NaCl	10% PEG	6.6	-
58	2.5% PEG 8000	15% PEG	6.6	-
59	1.4% PEG 8000	10% PEG	6.6	-
60	4% PEG 8000	8% PEG	6.6	-
61	5% PEG 8000	10% PEG	6.6	-
62	3.3% PEG 8000	6% PEG	6.6	-
63	2.5% PEG 8000	5% PEG	6.6	-
64	2% PEG 8000	4% PEG	6.6	-
65	1.6% PEG 8000	3% PEG	6.6	-
66	5% PEG 8000, 1 M NaCl	10% PEG	6.6	-
67	5% PEG 8000, 1.5 M NaCl	10% PEG	6.6	-
68	5% PEG 8000, 1.5 M NaCl	10% PEG	6.0	-
69	5% PEG 8000, 1.5 M NaCl	10% PEG	5.4	-
70	2% PEG 8000, 1.5 M NaCl	5% PEG	6.6	-
71	2% PEG 8000, 1.5 M NaCl	5% PEG	6.0	-
72	2% PEG 8000, 1.5 M NaCl	5% PEG	5.4	-
73	5% PEG 8000, 0.5 M NaCl	10% PEG	6.6	-
74	4% PEG 20000	6% PEG	6.6	-
75	2.86% PEG 20000	5% PEG	6.6	-
76	2% PEG 20000	4% PEG	6.6	-
77	1.4% PEG 20000	3% PEG	6.6	-
78	0.5 M citrate	1 M citrate	6.0	-
79	0.75 M citrate	1 M citrate	6.0	-
80	2M phosphate	2.5 M phosphate	7.0	-
81	2M phosphate	3 M phosphate	7.0	-
82	2M phosphate	2.2M phosphate	6.6	-
83	1.8 M phosphate	2.2M phosphate	6.6	-
84	1.8 M phosphate	2.2M phosphate	7.0	-
85	2M phosphate	2.2M phosphate	7.4	-
86	1.8 M phosphate	2.2M phosphate	7.4	-
87	30% ethanol	50% ethanol	7.0	poor crystals
88	35% ethanol	50% ethanol	7.0	-
89	25% ethanol	50% ethanol	7.8	needles
90	25% ethanol	50% ethanol	7.4	needles
91	25% ethanol	50% ethanol	7.0	-
92	25% ethanol	50% ethanol	6.6	needles

Table 1 (cont.)

no.	drop	reservoir	pH	results
93	25% ethanol	50% ethanol	6.2	-
94	25% ethanol	50% ethanol	5.8	-
95	25% ethanol	50% ethanol	5.4	-
96	20% ethanol	30% ethanol	7.0	-
97	10% ethanol	20% ethanol	7.0	-
98	1 M sodium sulfate	1.5 M sodium sulfate	7.0	-
99	1.3 M sodium sulfate	2.6 M sodium sulfate	7.0	-
100	1.1 M sodium sulfate	2M sodium sulfate	7.0	-
101	1.1 M sodium sulfate	1.5 M sodium sulfate	7.0	-
102	30% isopropanol	50% isopropanol	6.6	-
103	30% isopropanol	50% isopropanol	7.2	-
104	30% isopropanol	50% isopropanol	7.4	-
105	30% isopropanol	50% isopropanol	7.8	-

AS = ammonium sulfate

PEG = polyethylene glycol

MPD = methylpentane diol

RT = room temperature

LT = low temperature

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